Veterinary Parasitology 169 (2010) 226–231

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Rapid communication

Rhinitis and disseminated disease in a ferret (*Mustela putorius furo*) naturally infected with *Sarcocystis neurona*

Ann P. Britton [a], J.P. Dubey [b,*], Benjamin M. Rosenthal [b]

[a] *Animal Health Centre, British Columbia Ministry of Agriculture and Lands, 1767 Angus Campbell Road, Abbotsford, BC V3G 2M3, Canada*
[b] *US Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Beltsville, MD 20705, USA*

ARTICLE INFO

Article history:
Received 29 October 2009
Received in revised form 16 December 2009
Accepted 30 December 2009

Keywords:
Rhinitis
Meningoencephalitis
Inhalation
Ferret
Olfactory nerve
Sarcocystis neurona
Equine protozoal myeloencepahlitis
Mustela putorius furo

ABSTRACT

Naturally occurring *Sarcocystis neurona* infection in a ferret (*Mustela putorius furo*) with rhinitis and disseminated disease are described for the first time. The ferret exhibited severe rhinitis with intra-lesional *S. neurona* merozoites and schizonts. Diagnosis was confirmed immunohistochemically by staining with *S. neurona*-specific antibodies, and by phylogenetic analyses of conserved and variable portions of nuclear ribosomal DNA. On the basis of intense schizogony in the nasal mucosa, we propose the possibility of an olfactory nerve pathway route of infection for *S. neurona* meningoencephalitis.

1. Introduction

Equine protozoal myeloencephalitis (EPM) associated with the apicomplexan, *Sarcocystis neurona,* is a major cause of neurological disease in horses (Dubey et al., 2001a). *S. neurona* is transmitted via the fecal oral route from opossums, the only known definitive host, to an unusually wide array of intermediate hosts including raccoons, armadillos, cats, marine mammals, skunks and brown-headed cowbirds (Forest et al., 2000; Dubey et al., 2006; Mansfield et al., 2008). *S. neurona* infection in association with EPM-like disease has been reported in a diverse range of other wild and domestic mammals, including Pacific harbor seals, sea otters, a Canada lynx, mink, domestic cats and dogs, raccoons, striped skunks, and a fisher (reviewed in Dubey et al., 2006).

Naturally acquired *S. neurona* infection is typically localized to the central nervous system, reflecting an affinity of the organism for neural tissue (Dubey et al., 2001a). Disseminated disease, involving the brain and numerous other organs, has not been reported in naturally infected animals, although organisms were observed in the heart of a naturally infected raccoon (Hamir and Dubey, 2001). Experimentally, disseminated disease can be induced by oral inoculation of immunosuppressed mice with *S. neurona* sporocysts (Dubey, 2001) or by feeding large numbers of sporocysts to immunocompetent raccoons (Dubey et al., 2001b; Stanek et al., 2002).

In this paper, we describe disseminated disease including rhinitis in a ferret (*Mustela putorius furo*) naturally infected with *S. neurona*. To our knowledge, this is the first report of naturally acquired *S. neurona* rhinitis and disseminated disease in any species.

2. Materials and methods

2.1. Naturally infected ferret

A male, juvenile domestic ferret weighing 374 g was presented to the Animal Health Centre (AHC), Abbotsford,

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
E-mail address: jitender.dubey@ars.usda.gov (J.P. Dubey).

0304-4017/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2009.12.043

British Columbia, Canada on October 1, 2008 for necropsy. The ferret had been purchased from a pet store in Nanaimo, British Columbia. One dose of modified live virus (MLV) canine distemper virus (CDV) vaccine had been administered to the ferret prior to arrival at the pet store. Within one week of purchase, the ferret developed a nasal discharge, respiratory disease, dehydration and hind end paresis. Due to the poor prognosis, the ferret was euthanized.

2.2. Immunohistochemical examination

Tissue samples were fixed in 10% buffered formalin, routinely processed, sectioned at 3–5 μm and stained with hematoxylin and eosin for histopathology. Immunohistochemistry (IHC) for CDV was conducted at AHC on paraffin-embedded tissues using the labelled streptavidin–biotin alkaline phosphastase method and the Ventana Nexes automated stainer (Tucson, AZ, USA) as previously described (Haines and Chelak, 1991) employing a polyclonal rabbit anti-measles virus primary antibody.

Immunohistochemistry for *S. neurona*, *Toxoplasma gondii*, and *Neospora caninum* was performed at the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture, Beltsville, MD, using reagents and methods described previously (Lindsay and Dubey, 1989; Dubey et al., 1999; Dubey and Hamir, 2000).

2.3. Molecular examination

Unfixed, tissue samples from brain, lung, liver, kidney and spleen were pooled and analyzed by RT-PCR for the CDV phosphoprotein (P) gene. External primers 5′-GAAGATGCTGACAGT CTCGTG-3′ and 5′-CAACTATCCC-CATTCCATGTG-3′ identifying a 364 bp product were employed in the first round. Nested primers 5′-TCTGGCGAAGATTATTCCG-3′ and 5′-TCCCTACATTTCTGCT-TGTCC-3′ were employed in the second round, yielding a 142 bp product.

To diagnose the parasitic agent, pooled, frozen, unfixed tissues of the ferret were processed at APDL. A 982 bp portion of parasite 18S rDNA was amplified using primers 18S1F and 18S11R (Rosenthal et al., 2008) and the complete first internal transcribed ribosomal spacer (ITS-1) was amplified using primers situated in the flanking 18S and 5.8S rDNA: 18S-14F 5′-GTTGGTTTCTAGGACTGA-3′ and 5.8S-2R 5′-TTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGA-3′. BLAST analysis was used to identify similar sequences in

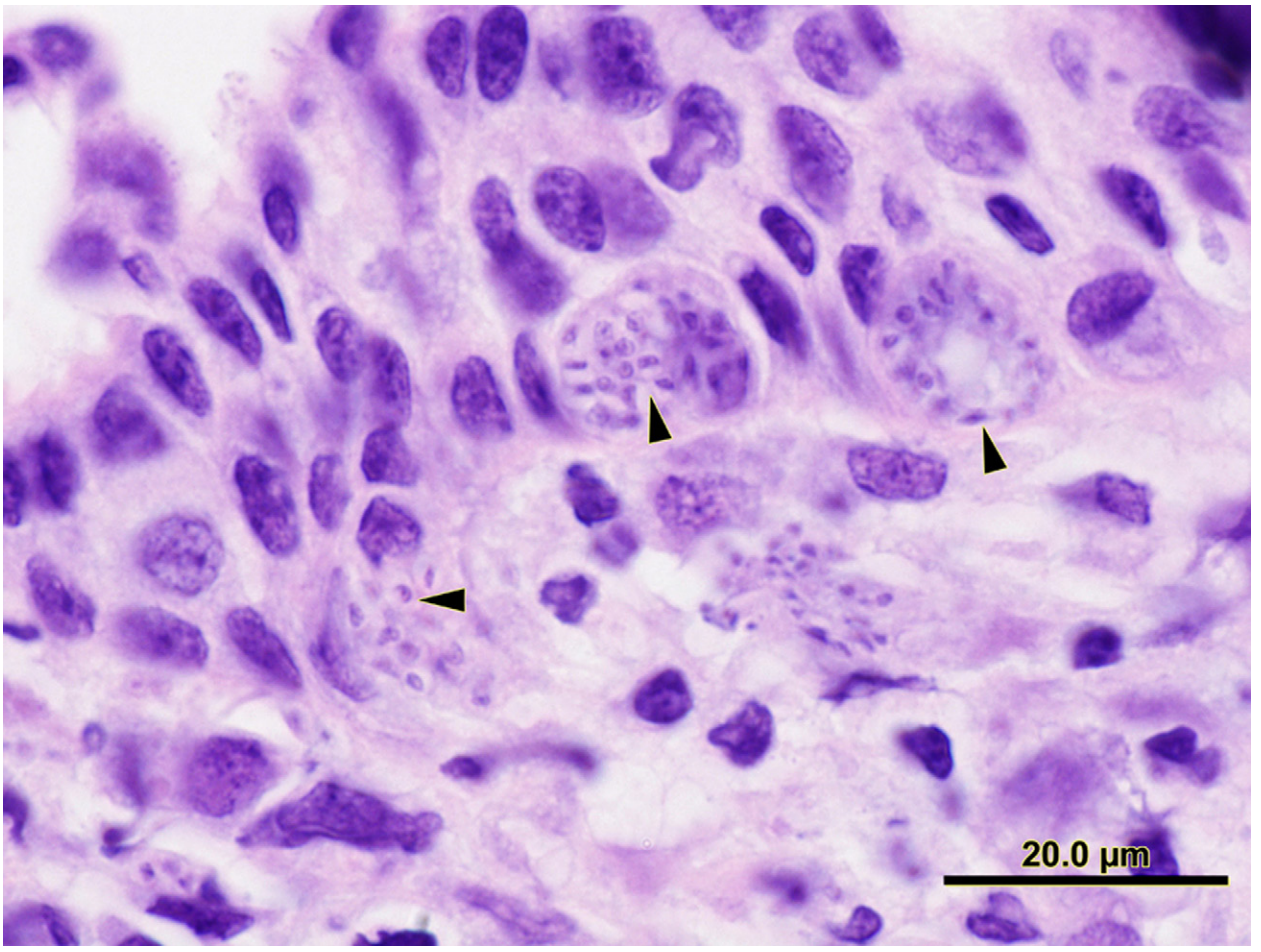

Fig. 1. Nasal mucosa. Schizonts of *S. neurona* are present within epithelial cells (arrowheads) lining the nasal mucosal surface. H&E.

GenBank, and MUSCLE (Edgar, 2004) was used to construct a multiple sequence alignment prior to phylogenetic reconstruction under the criterion of minimum evolution using the maximum composite likelihood method as implemented by MEGA4 after complete deletion of gapped positions (Tamura et al., 2007).

3. Results

3.1. Lesions and immunohistochemistry

Gross findings were restricted to the respiratory tract. The ferret exhibited mucopurulent exudate around the nares and over the mucosal surface of the turbinates. The lungs were diffusely firm, dark red to purple in color with pinpoint, pale tan foci randomly scattered over the pleural surface.

Microscopically, moderate, multifocal, mixed inflammatory cell infiltrates were observed within the nasal lamina propria with a suppurative exudate along the mucosal surface. Mucosal epithelium was disrupted by cell swelling and infiltration of inflammatory cells. Schizonts could be observed within both mucosal epithelium and lamina propria in H&E stained sections (Fig. 1).

The organism reacted positively to antibodies with affinity for *S. neurona* but not to antibodies specific to either *T. gondii* or *N. caninum*. With the aid of IHC, numerous *S. neurona* schizonts and merozoites were demonstrated in the lamina propria, most intensely congregated around the turbinate bones (Fig. 2). Both *S. neurona* schizonts and zoites were observed in the mucosal epithelium, often associated with thickened areas compatible with olfactory epithelium, and occasionally within glandular epithelium (Fig. 2, inset). Throughout the remainder of the tissues, IHC revealed *S. neurona* merozoites, and to a lesser extent schizonts, in lung, brain, heart, skeletal muscle, adrenal gland, liver, spleen, lymph nodes, kidney, skin and intravascular monocytes. Within the lung, multifocal to coalescing areas of alveolar consolidation, characterized by macrophages and lymphocytes, were associated with moderate numbers of schizonts and merozoites. Moderate numbers of schizonts and merozoites were also observed in the brain, with multifocal, perivascular mixed inflammation at all levels

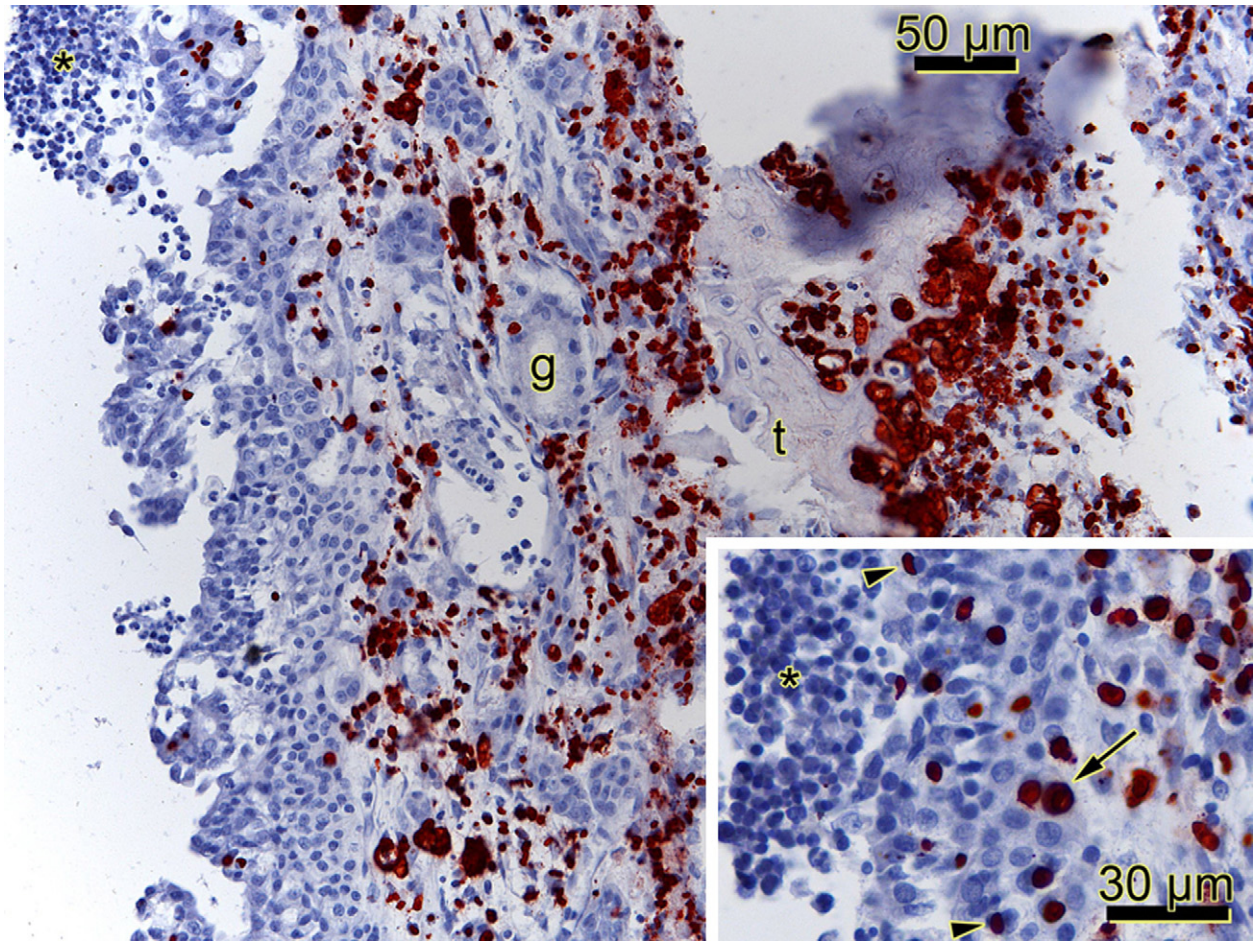

Fig. 2. Nasal mucosa. Large numbers of schizonts and zoites can be observed within the lamina propria and epithelium as a result of positive immunohistochemical staining for *S. neurona*. Note the marked intensity of organisms around turbinate bone (t), zoites within glandular epithelium (g) and the inflammatory exudate on the mucosal surface (*). *Inset*: Higher power of adjacent mucosal epithelium demonstrating intraepithelial zoites (arrowheads) and schizonts (arrow).

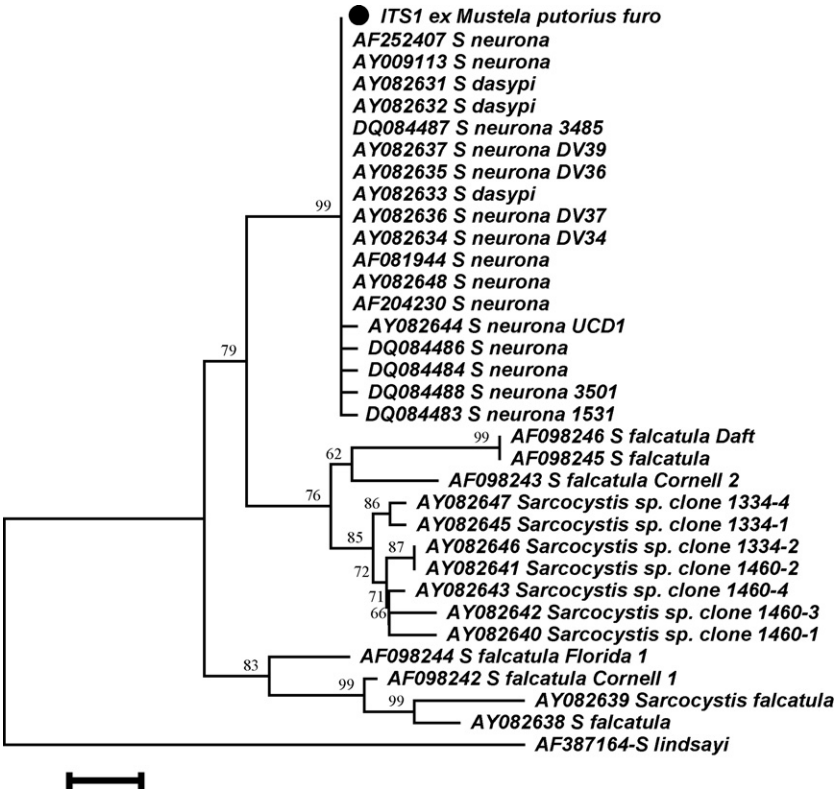

Fig. 3. Minimum evolution tree reconstructed from ITS-1. The percentage of replicate trees in which the associated taxa clustered together in 500 bootstrap replicates is shown. Branch lengths are proportional to evolutionary distances computed using the maximum composite likelihood method, measured as the number of base substitutions per site. After complete deletion of gapped positions, 940 bases were used in this analysis.

and moderate multifocal gliosis largely in grey matter. Lesser numbers of organisms were associated with mild to moderate mixed inflammatory cell infiltrates in the heart, skeletal muscle, adrenal gland, liver and skin. Few schizonts and zoites were observed in the gastrointestinal epithelium. Marked thymic and moderate nodal and splenic lymphoid depletion was observed. Mid-shaft femoral bone marrow was highly cellular with all cell lines represented and an increase in the myeloid to erythroid ratio.

3.2. CDV examination

Polymerase chain reaction assay for CDV was positive on pooled tissues. Immunohistochemistry for CDV was negative on lung, urinary bladder, thymus, brain and kidney. No epithelial inclusion bodies of CDV were observed in any tissues.

3.3. Molecular systematics

The parasite PCR product was 1097 bp. BLAST analyses identified several sequences closely related to those amplified from the present case, all derived either from *S. neurona* or from parasites which may or may not have been accurately distinguished from *S. neurona* on the basis of host associations or morphological attributes. Phylogenetic analyses of the more discriminatory ITS-1 sequences consistently differentiated the present isolate from representatives of the closely related taxon *S. falcatula,* instead placing them in an invariable clade comprised only of several isolates of *S. neurona* and to certain parasites, derived from nine-banded armadillos, attributed to *S. dasypi* (Fig. 3). In spite of the nomenclatural uncertainty arising from the lack of genetic distinction evident among several parasite lineages that are currently recognized as distinct taxa, the available data are entirely consistent with the diagnosis of *S. neurona* in this case.

4. Discussion

Disease associated with *S. neurona* is rarely observed outside the central nervous system in naturally infected animals (Dubey et al., 2001a). This case is exceptional among naturally acquired infections because meningoencephalitis was accompanied by severe disseminated disease, including marked rhinitis. Meningoencephalitis with disseminated disease can be experimentally produced in gamma interferon knockout mice (Dubey, 2001), but not in immunocompetent mice (Marsh et al., 1997) via oral inoculation suggesting that immunosuppression likely facilitates extra-neural dissemination of *S. neurona*. Co-infection with CDV in raccoons suffering from *S. neurona* meningoencephalitis has been reported (Stoffregen and Dubey, 1991; Thulin et al., 1992) and immunosuppression by CDV was thought to have predisposed these animals to

S. neurona infection. Canine distemper virus infection causes severe lymphopenia (Kauffman et al., 1982; Von Messling et al., 2004) coincident with lymphoid depletion in the thymus, spleen and lymph nodes (Dungworth, 1993).

Ferrets are exquisitely susceptible to CDV infection, which is highly immunosuppressive and 100% fatal in this species (Fox et al., 1998; Williams et al., 1988). The ferret in this study did not have CDV disease. However, the ferret had been vaccinated with a modified live CDV vaccine and exhibited significant depletion of lymphocytes in the thymus, spleen and lymph nodes, changes associated with CDV immunosuppression (Dungworth, 1993). Lymphopenia similar to natural infection can be produced by MLV CDV vaccines (Kauffman et al., 1982) and ferrets are reported to be susceptible to secondary infections during the post-vaccinal period of lymphoid depletion (Williams et al., 1996). Immunosuppression due to MLV CDV vaccination can also predispose dogs to toxoplasmosis (Dubey et al., 2003). Thus, it is probable that post-vaccinal immunosuppression predisposed the ferret to *S. neurona* infection.

Rhinitis with intense schizogony was observed in the ferret. Rhinitis has not been reported previously in association with natural or experimental *S. neurona* infection in any host. The marked schizogony in the nasal mucosa was unparalleled in any other organ, suggesting that nasal mucosa may be a predilection site for *S. neurona*. Unlike most other species of *Sarcocystis*, asexual reproduction of *S. neurona* is not commonly observed in endothelium; instead, schizonts are more often observed within neurons and monocytes, a finding compatible with the affinity of *S. neurona* for the central nervous system (Dubey et al., 2001a; Dubey, 2001). Olfaction is the primary function of the nasal cavity in carnivores, such as the ferret. Consequently, large areas of the nasal mucosal surface are devoted to olfactory epithelium, a specialized, thickened structure comprised of olfactory neurons continuous with nerve bundles in the lamina propria. These run through the cribriform plate to the olfactory bulb of the brain (Reznik, 1990). The intense population of schizonts and merozoites associated with the nasal mucosa in the ferret may thus reflect an affinity of *S. neurona* for olfactory neurons.

An olfactory nerve pathway route of infection is well established for a number of pathogens which cause encephalitis, such as CDV (Rudd et al., 2006) and the free living amoebae, *Naegleria fowleri* and *Balamuthia mandrillaris* (Jarolim et al., 2000; Kiderlen and Laube, 2004). While CDV is distributed to the olfactory nerves hematogenously (Rudd et al., 2006), *N. fowleri* and *B. mandrillaris* infect the olfactory neurons directly after nasal mucosal invasion. It is unclear whether *S. neurona* entered the brain via the olfactory nerve pathway in the ferret. Large numbers of merozoites were present in the nasal lamina propria, but whether these were invading olfactory nerve bundles was difficult to determine due the small size of the bundles and the marked degree of inflammation.

S. neurona schizonts and merozoites were observed in the nasal epthelium of the ferret. Entry of *S. neurona* via the nasal mucosal epithelium would afford direct access to a large number of olfactory nerves leading directly to the brain. On the basis of the rhinitis with intense schizogony observed in this ferret, intranasal inoculation studies and further investigation of both a nasal epithelial portal of entry and an olfactory nerve pathway route of infection for *S. neurona* is warranted.

Acknowledgements

We would like to thank Dr. Gary Marty for cheerfully providing technical assistance with photographs, Sandra Etheridge for her expertise in slide preparation and immunohistochemistry and Julie Bidulka and Detiger Dunams for performing PCR assays. Dr. Ziola of the University of Saskatchewan, College of Medicine kindly provided the polyclonal rabbit anti-measles virus primary antibody.

References

Dubey, J.P., Mattson, D.E., Speer, C.A., Baker, R.J., Mulrooney, D.M., Tornquist, S.J., Hamir, A.N., Gerros, T.C., 1999. Characterization of *Sarcocystis neurona* isolate (SN6) from a naturally infected horse from Oregon. J. Eukaryot. Microbiol. 46, 500–506.

Dubey, J.P., Hamir, A.N., 2000. Immunohistochemical confirmation of *Sarcocystis neurona* infections in raccoons, mink, cat, skunk, and pony. J. Parasitol. 86, 1150–1152.

Dubey, J.P., 2001. Migration and development of *Sarcocystis neurona* in tissues of interferon gamma knockout mice fed sporocysts from a naturally infected opossum. Vet. Parasitol. 95, 341–351.

Dubey, J.P., Lindsay, D.S., Saville, W.J.A., Reed, S.M., Granstrom, D.E., Speer, C.A., 2001a. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Vet. Parasitol. 95, 89–131.

Dubey, J.P., Saville, W.J.A., Stanek, J.F., Lindsay, D.S., Rosenthal, B.M., Oglesbee, M.J., Rosypal, A.C., Njoku, C.J., Stich, R.W., Kwok, O.C.H., Shen, S.K., Hamir, A.N., Reed, S.M., 2001b. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): evidence for natural infection with sarcocysts, transmission of infection to opossums (*Didelphis virginiana*), and experimental induction of neurologic disease in raccoons. Vet. Parasitol. 100, 117–129.

Dubey, J.P., Ross, A.D., Fritz, D., 2003. Clinical *Toxoplasma gondii*, *Hammondia heydorni*, and *Sarcocystis* spp. infections in dogs. Parasitologia 45, 141–146.

Dubey, J.P., Chapman, J.L., Rosenthal, B.M., Mense, M., Schueler, R.L., 2006. Clinical *Sarcocystis neurona*, *Sarcocystis canis*, *Toxoplasma gondii*, and *Neospora caninum* infections in dogs. Vet. Parasitol. 137, 36–49.

Dungworth, D.L., 1993. The respiratory system. In: Jubb, K.V.F., Kennedy, P.C., Palmer, N. (Eds.), Pathology of Domestic Animals, 4th ed., 2. Academic Press, Toronto, pp. 617–624.

Edgar, Robert C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucl. Acid. Res. 32, 1792–1797.

Forest, T.W., Abou-Madi, N., Summers, B.A., Tornquist, S.J., Cooper, B.J., 2000. *Sarcocystis neurona*-like encephalitis in a Canada lynx (*Felis lynx canadensis*). J. Zoo Wild. Med. 31, 383–387.

Fox, J.G., Pearson, R.C., Gorham, J.R., 1998. Viral diseases. In: Fox, J.G. (Ed.), Biology and Disease of the Ferret. 2nd ed. Wiley-Blackwell, Ames, IA, p. 356.

Haines, D.M., Chelak, B.J., 1991. Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. J. Vet. Diagn. Invest. 3, 101–112.

Hamir, A.N., Dubey, J.P., 2001. Myocarditis and encephalitis associated with *Sarcocystis neurona* infection in raccoons (*Procyon lotor*). Vet. Parasitol. 95, 335–340.

Jarolim, K.L., McCosh, J.K., Howard, M.J., John, D.T., 2000. A light microsocopy study of the migration of *Naegleria fowleri* from the nasal submucosa to the central nervous system during the early stage of primary amebic meningoencephalitis in mice. J. Parasitol. 86, 50–55.

Kauffman, C.A., Bergman, A.G., O'Connor, R.P., 1982. Distemper virus infection in ferrets: an animal model of measles-induced immunosuppresssion. Clin. Exp. Immunol. 47, 617–625.

Kiderlen, A.F., Laube, U., 2004. *Balamuthia mandrillaris*, an opportunistic agent of granulomatous amebic encephalitis, infects the brain via the olfactory nerve pathway. Parasitol. Res. 94, 49–52.

Lindsay, D.S., Dubey, J.P., 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am. J. Vet. Res. 50, 1981–1983.

Mansfield, L.S., Mehler, S., Nelson, K., Elsheikha, H.M., Murphy, A.J., Knust, B., Tanhauser, S.M., Gearhart, P.M., Rossano, M.G., Bowman, D.D., Schott, H.C., Patterson, J.S., 2008. Brown-headed cowbirds (*Molothrus ater*) harbor *Sarcocystis neurona* and act as intermediate hosts. Vet. Parasitol. 153, 24–43.

Marsh, A.E., Barr, B.C., Lakritz, J., Nordhausen, R., Madigan, J.E., Conrad, P.A., 1997. Experimental infection of nude mice as a model for *Sarcocystis neurona*-associated encephalitis. Parasitol. Res. 83, 706–711.

Reznik, G.K., 1990. Comparative anatomy, physiology, and function of the upper respiratory tract. Environ. Health Perspect. 85, 171–176.

Rosenthal, B.M., Dunams, D.B., Pritt, B., 2008. Restricted genetic diversity in the ubiquitous cattle parasite, *Sarcocystis cruzi*. Infect. Gen. Evol. 8, 588–592.

Rudd, P.A., Cattaneo, R., von Messling, V., 2006. Canine distemper virus uses both the anterograde and the hemaotogenous pathway for neuroinvasion. J. Virol. 80, 9361–9370.

Stanek, J.F., Dubey, J.P., Oglesbee, M.J., Reed, S.M., Lindsay, D.S., Capitini, L.A., Njoku, C.J., Vittitow, K.L., Saville, W.J.A., 2002. Life cycle of *Sarcocystis neurona* in its natural intermediate host, the raccoon, *Procyon lotor*. J. Parasitol. 88, 1151–1158.

Stoffregen, D.A., Dubey, J.P., 1991. A *Sarcocystis*-like protozoan and concurrent canine distemper virus infection associated with encephalitis in a raccoon (*Procyon lotor*). J. Wild. Dis. 27, 688–692.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thulin, J.D., Granstrom, D.E., Gelberg, H.B., Morton, D.G., French, R.A., Giles, R.C., 1992. Concurrent protozoal encephalitis and canine distemper virus infection in a raccoon (*Procyon lotor*). Vet. Rec. 130, 162–164.

Von Messling, V., Milosevic, D., Cattaneo, R., 2004. Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. Proc. Natl. Acad. Sci. U.S.A. 101, 14216–14221.

Williams, E.S., Thorne, E.T., Appel, M.J.G., Belitsky, D.W., 1988. Canine distemper in black-footed ferrets (*Mustela nigripes*) from Wyoming. J. Wild. Dis. 24, 385–398.

Williams, E.S., Anderson, S.L., Cavender, J., Lynn, C., List, K., Hearn, C., Appel, M.J.G., 1996. Vaccination of black-footed ferret (*Mustela nigripes*) × Siberian polecat (*M. eversmanni*) hybrds and domestic ferrets (*M. putorius furo*) against canine distemper. J. Wild. Dis. 32, 417–423.